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Evaluation of some properties of a phosphorodithioate oligodeoxyribonucleotide for antisense application

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ABSTRACT

An all phosphorodithioate oligodeoxyribonucleotide (PS_2; 17-mer) complementary to the coding region of the rabbit β-globin mRNA was compared with the normal (PO_2) and phosphorothioate (POS) oligonucleotide of the same size and sequence with respect to physicochemical properties and antisense activity in cell-free systems. The melting temperature (Tm) of the PS_2–cDNA duplex was reduced by 17°C relative to the PO_2–cDNA duplex, compared to 11°C for the POS–cDNA duplex, suggesting a decreased stability of the duplex with an increasing sulfur substitution. Like the POS-derivative, the PS_2 oligonucleotide is quite stable against exonucleases, but these modified oligonucleotides showed different stability towards endonucleases and also towards different sub-cellular fractions of MCF-7 cells. During *in vitro* protein binding studies, the PS_2 oligonucleotide showed similar binding (10–20%) to that of the PO_2 oligonucleotide, while the POS oligonucleotide bound 60%. In cell-free translation, the PS_2 oligonucleotide produced slightly higher specific translation inhibition of rabbit β-globin mRNA compared to that of the PO_2 oligonucleotide, and this was true only at concentration below 2 mM. The POS-derivative, except at 10 mM concentration, always showed higher translation arrest of the rabbit β-globin mRNA compared to that of the other two oligonucleotides. The present study suggests that the PS_2 oligonucleotide offers very little advantage over the POS oligonucleotide for use as an antisense analog.

INTRODUCTION

Antisense oligonucleotides have great potential in biomedical sciences because of their ability to interact with RNA and DNA under physiological conditions. By interaction with complementary DNA or RNA, they can control gene expression either by inhibiting translation (1,2) or transcription (3). The therapeutic value of normal oligonucleotides, however, is seriously hampered because of their susceptibilities towards different nucleases present in cells and serum (4–7). Improvement has been made by chemically modifying the phosphate backbone, and several classes of phosphate-modified oligonucleotides have been synthesized.

Among the chemically modified analogues, phosphorothioates and methylphosphonates have been widely investigated and studied (8–17). In contrast to normal oligonucleotides, these oligonucleotides posses a chiral phosphorus atom which inevitably gives rise to a number of nonresolvable diastereomeric products that offer different nuclease stability (18), and form duplexes with RNA of variable stability. The problem of chirality in such synthetic modified oligodeoxyribonucleotides can be overcome by substituting both the non-bridging oxygen atoms in the phosphate backbone with the same atom. The phosphorodithioate (PS_2) oligonucleotides, where both oxygen atoms are substituted by sulfur atoms, are very promising since they are achiral and isopolar with the PO_2 oligonucleotides, are chemically very stable, can easily be alkylated (19) and are stable towards exonucleases (20). They were also found very potent compared to other oligonucleotides in inhibiting both avian and human immunodeficiency virus type-1 reverse transcriptase (21,22). Despite such improved properties, very few studies have been reported with respect to essential features required for antisense application of this analog. Most of the studies reported on PS_2 oligonucleotides deal with the synthesis of deoxynucleoside phosphorodithioate dimers using phosphorodiamidite (19, 20,23,24), thiophosphoramidite (24–27), H-phosphonothioate (28–30), H-phosphonodithioate (31–34) or phosphorodithioate monomers (35,36). In order to consider PS_2 oligonucleotides as potentially useful for antisense applications, a detailed study of their physicochemical properties and evaluation of their antisense activities are needed. In a recent paper, Caruthers *et al.* (20) stated 'if these observations (stability against snake venom and spleen phosphodiesterase) can be extended to other exo- and endonucleases as well, phosphorodithioate analogs may be very useful for various diagnostic and therapeutic applications'. The present investigation has been directed to determine such properties using a 17-mer all-PS_2 sequence complementary to the coding region of the rabbit β-globin mRNA. Earlier results showing translation arrest of this mRNA in cell-free systems by both normal (PO_2) and phosphorothioate (POS) oligonucleotides (7,9,37,38) have led us to use the same sequence in the present investigation.

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MATERIALS AND METHODS

The enzymes like S1, P1, snake venom phosphodiesterase (SPD), bovine spleen phosphodiesterase (BPD), deoxyribonuclease I (DNase I), and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories (BRL, Rockville, MD) and Sigma Chemical Company (St Louis, MO). RNase H (from *E.coli*) and rabbit globin mRNA were also obtained from BRL. The cell-free systems wheat germ agglutinin (WGA), rabbit reticulocyte lysate (RRL), brome mosaic virus (BMV) mRNA and all other ingredients necessary for cell-free translation were obtained from Promega corporation (Madison, WI). L-[^{35}S] methionine (1128.5 Ci/mmol) and [γ-^{32}P] adenosine 5'-triphosphate triethylammonium salt (3000 Ci/mmol) were obtained from NEN research products. Human RNase H1 was a kind gift from Dr J.Walder (University of Iowa). All other ingredients used during this study were of analytical grade.

Oligodeoxynucleotide synthesis

The synthesis of 17-mer oligodeoxyribonucleotides 5'-d(CACC-AACTTCTTCCACA), containing PO_2 and POS linkages, as well as the complementary 17-mer unmodified oligodeoxyribo-nucleotide, were described earlier (38). The PS_2 oligonucleotide was synthesized by a thiophosphoramidite method and purified as described before (39). It contained 8% of phosphorothioate linkages according to ^{31}P NMR.

Labeling of oligonucleotides

Except for the PS_2-derivative, the labeling of oligonucleotides at their 5'-terminal end was carried out in the same way as described earlier (38). Labeling of PS_2 was carried out at 37°C with three times addition of twice the enzyme concentration over a period of 3 hrs, but that still results in lower incorporation of [^{32}P] compared to the two other oligonucleotides.

Analysis of nuclease-digested products

The enzyme-hydrolyzed products of ^{32}P labeled oligonucleotides was monitored by autoradiography as described earlier (38).

Melting temperature/nuclease digestion/protein binding/activation of RNase H (H1)/*in vitro* translation

All of these procedures used for the determination of the properties of the PS_2-derivative were the same as those described previously (38).

Stabilities against deoxyribonuclease I

The reaction was carried out in 50 mM sodium acetate buffer (pH 6.5) containing 10 mM $MgCl_2$, 2 mM $CaCl_2$ and [^{32}P] oligonucleotides (mixed with 5 nmol of cold oligo) in a total volume of 0.2 ml. The reaction was initiated with the addition of 50 units of enzyme, and the mixtures were incubated at 37°C for several minutes. At predetermined times, an aliquot (0.02 ml) was withdrawn from the reaction mixture and mixed thoroughly with an equal volume of 9 M urea in 2×TBE (0.2 M Tris−HCl, pH 8.3, 0.2 M sodium borate and 4 mM EDTA). The samples were frozen immediately and kept at −70°C until electrophoresis was carried out.

Stability in subcellular fraction of MCF-7 cells

Cell fractionation was carried out as described by Hoke *et al.* (40), and the nuclear fraction was checked by microscopic visualization. Labeled oligonucleotides (mixed with cold) were added with 0.1 ml of different fractions (100 mg of protein), and the volume was adjusted to 0.2 ml with 20 mM phosphate buffer (pH 7.0). The reaction mixtures were incubated at 37°C for several hours and the aliquots, withdrawn at different time points, were analyzed as described for DNase I.

Binding studies with wheat-germ agglutinin components

Ten microliters (0.01 ml) of ^{32}P end-labeled oligonucleotides were incubated at 37°C for 4 hrs with 0.09 ml of cell-free extract. In control experiments, the volumes were adjusted with equal amounts of buffer (PBS; 0.025 M phosphate buffer, pH 7.2 containg 0.9% NaCl). Following incubation, the samples were filtered through ultrafree-MC filter units (30,000 and 10,000 NMWL, PLTK and PTGC respectively) by centrifugation (Microfuge 12) at 12000 r.p.m for 5−10 min to seperate the unbound oligo. The counts present in the samples and the controls (before and after filtration) were used to calculate the percent bound oligonucleotide with the protein factors in the cell-free extract.

RESULTS

Melting temperatures

The PS_2 oligomer formed a duplex with the complementary unmodified oligodeoxyribonucleotide (cDNA), but the duplex was somewhat less stable than that of the POS-derivative as seen from the decrease in melting temperature (Figure 1). The Tm value was reduced by 17°C relative to the natural oligo, corresponding to 1° depression per modified base.

Protein-binding studies

Protein-binding studies with HSA showed that the PS_2 and PO_2 oligonucleotide was bound to the same extent (10−20%), whereas 60% of the POS-derivative was bound. In the absence of protein, very little (0−4%) of the oligomers were bound to

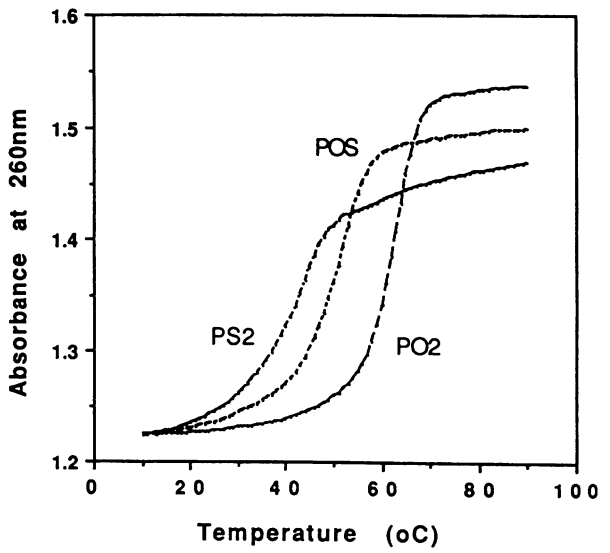

Figure 1. Melting temperature curves of the oligonucleotides hybridized to their complementary 17-mer DNA strand. Equimolar amounts of oligonucleotides were preheated at 75°C for 10 min in 10 mM sodium cacodylate buffer containing 0.14 M NaCl before measurements at 260 nm.

the membrane, and this amount was subtracted in the determination of percentage oligo bound with the protein.

Nuclease stabilities

The PS_2 oligonucleotide had a similar stability as that of the POS-derivative towards P1 nuclease, and different exonucleases such as BPD and SPD [figure not shown here because the stability pattern of the POS oligonucleotide was presented earlier (38)]. The PS_2-derivative, however, showed different resistance towards the hydrolytic attack of endonucleases. With S1 nuclease, only 8−10% of the PS_2-derivative was degraded over a period of 10 min compared to 25−30% degradation of the POS-derivative (Figure 2). In contrast, the PS_2-derivative was as unstable as the PO_2 oligonucleotide towards DNase I (Figs 3, 4). The half-lives ($t_{1/2}$) for cleavage by DNase I (calculated from the initial linear portion of the graphs) were found to be 60−65 min for both the PO_2, and PS_2 oligonucleotides.

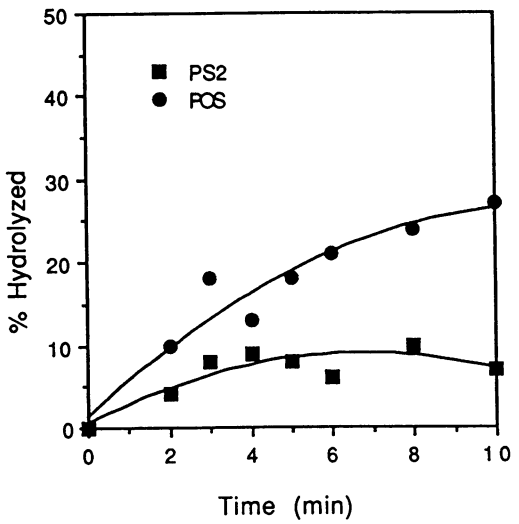

Figure 2. Stabilities of oligonucleotides towards S1 nuclease. The hydrolysis was carried out for 10 min at 37°C in 0.03 M sodium acetate buffer (pH 4.6) containing 0.05 M NaCl, 1 mM $Zn(OAc)_2$, 5% glycerol and ^{32}P-labeled oligonucleotides. The percent hydrolyzed was determined from a control experiment where enzyme was not added. The autoradiographs were scanned following electrophoresis as described in the text.

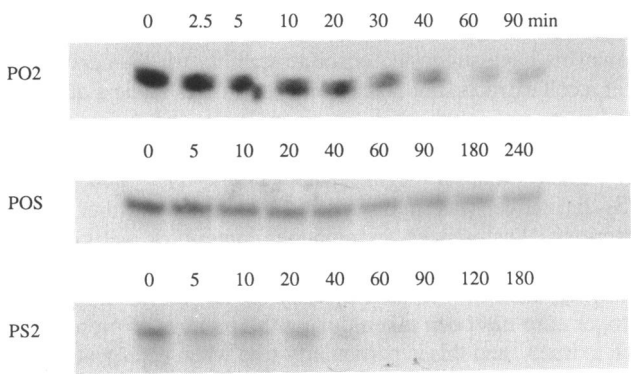

Figure 3. Stabilities of PO_2 (top), POS (middle) and PS_2 (bottom) oligonucleotides towards DNase I. The experiment was carried out as described in the text, and the aliquots for PO_2 were collected at 0, 2.5, 5, 10, 20, 30, 40, 60 and 90 min for enzyme digested products. Aliquots for POS and PS_2 oligonucleotides were collected at 0, 5, 10, 20, 40, 60, 90, 120 (for PS_2 only), 180 and 240 (for POS only) min.

Stabilities in subcellular fraction

In the presence of different sub-cellular fractions of MCF-7 cells, the modified oligonucleotides showed different degrees of resistance towards endogeneous nucleases present in the fractions (Figure 5a,b). The PO_2 oligonucleotide was completely degraded within 2−3 hr of incubation in the presence of these fractions, and the hydrolysis occured more rapidly in the post-nuclear fraction compared to the nuclear fraction. The hydrolysis of the modified derivatives, on the other hand, was faster in the nuclear fraction. For the PS_2-derivative, 50% was hydrolyzed within 2.5 and 5 hrs in the presence of the nuclear and post-nuclear fraction, respectively. For the same amount of degradation of the POS-derivative, it took 6 hrs (nuclear) or more (post-nuclear) suggesting a greater stability of the POS-derivative over the PS_2-derivative.

RNase H activity and translation in cell-free system

The degradation rate of the rabbit β-globin mRNA by both RNase H (*E.coli*) and RNase H1 (human) in the PS_2 oligonucleotide-mRNA duplex was very similar to that of the PO_2 oligomer−mRNA duplex, and 1.3 times less than that of the POS-derivative. For the PS_2-derivative, the degradation rate of the target mRNA was 5.9 and 12.1×10^{-4} (ΔAbs_{260nm}/min) for RNase H (*E.coli*) and RNase H1 (human), respectively. The difference in the cleavage rate of the target mRNA by RNase H in the presence of oligonucleotides may thus be responsible for producing different antisense activity between the oligonucleotides. The POS oligonucleotide, indeed, showed much higher specific inhibition of translation of rabbit β-globin mRNA compared to the other oligonucleotides during translation in cell-free system (Table I). Specific inhibition which we define as the difference in translation inhibition between the control (BMV

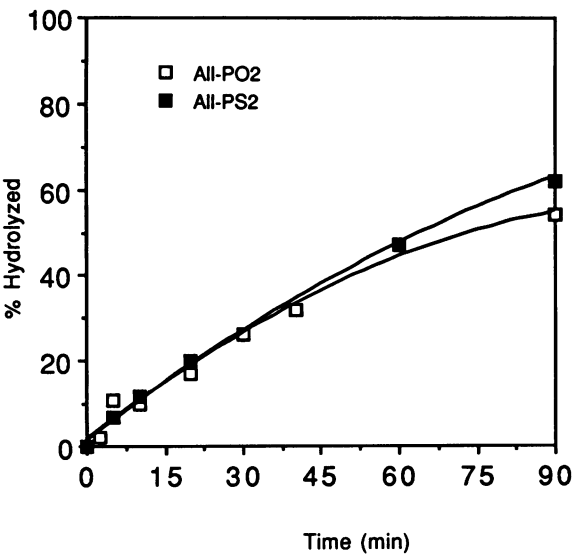

Figure 4. Stabilities of PO_2 and PS_2 oligonucleotides towards DNase I. The hydrolysis was carried out as described in the text, and the percent hydrolysis was determined from a control experiment where enzyme was not added. The autoradiographs were scanned following electrophoresis as described in the text.

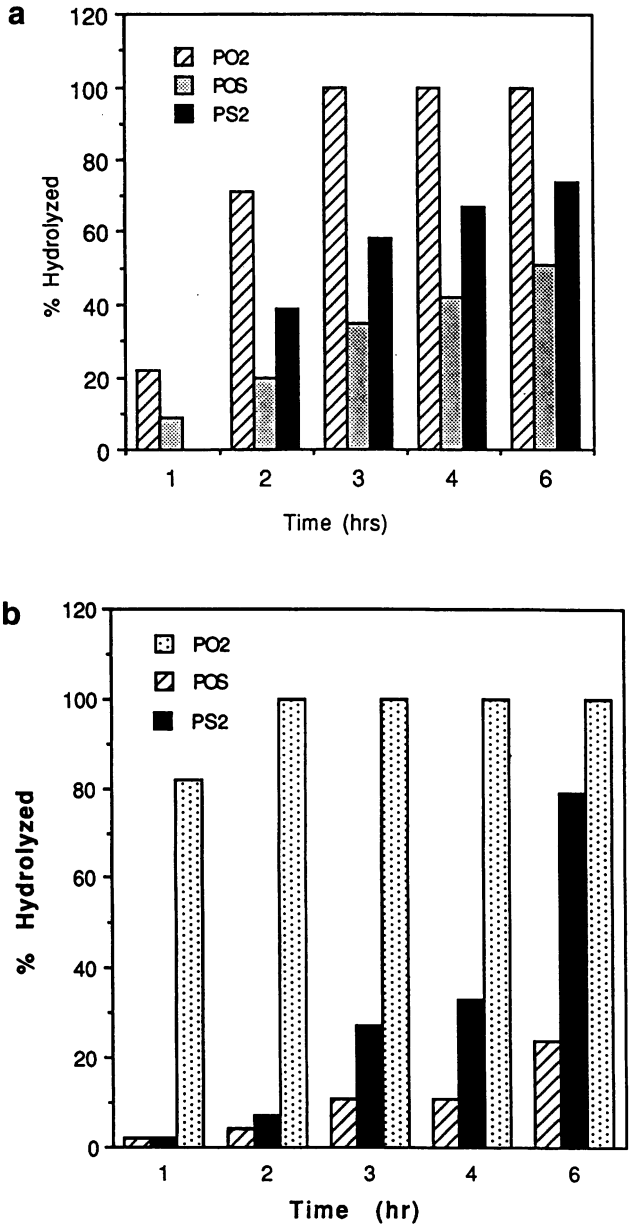

Figure 5. Stabilities of oligonucleotides in nuclear (a) and post-nuclear-nuclear fraction (b) of MCF-7 cells. The details of this procedure are described in the text, and the analysis of the autoradiographs following electrophoresis were carried out as described in the text.

mRNA) and the target mRNA, was almost 6 times higher compared to the PO_2-derivative and 2-6 times higher than the PS_2-derivative (Figure 6). With increased concentrations, however, PS_2 oligos showed an increased translation inhibition of the control mRNA, and thus results in a decrease of specific translation inhibition (Figure 7). In another cell-free system, i.e. RRL, neither of these showed any significant inhibition of translation unless RNase H was added exogeneously (data not shown).

DISCUSSION

Three 17-mer oligonucleotides, containing either contiguous natural (PO_2), phosphorothioate (POS) or phosphorodithioate (PS_2) linkages, complementary to the coding region of rabbit β-globin mRNA, were studied with respect to their physicochemical properties and antisense activity. The stability of the PS_2-derivative towards exonucleases and its reduced hybridizing ability with the complementary unmodified oligonucleotide are in good accordance with earlier results from different laboratories (20,30,39).

Table I. Concentration of oligonucleotides versus translation inhibition

Oligonucleotide	Conc. (μM)	Percent inhibition		
		BMV mRNA	Target mRNA	Specific inhibition
POS	0.5	12±3	73±1	61±2
	1.0	28±10	78±5	50±5
	2.0	31±8	75±0.5	44±7.5
	4.0	43±2	83±7	40±5
	10.0	86±1	99±1	13±0
PO_2	0.5	14±4	24±0	10±4
	1.0	16±5	24±1	8±4
	2.0	27±4	42±2	15±2
	4.0	22±2	36±8	14±6
	10.0	26±9	33±7	7±2
PS_2	0.5	30±6	40±8	10±2
	1.0	62±12	94±5	32±7
	2.0	74±18	98±2	24±16
	4.0	82±8	96±4	14±4
	10.0	92±4	98±2	6±2

The results of our hydrolytic studies with DNase I unexpectedly revealed that the PS_2-derivative was as unstable as the PO_2 oligonucleotide. Varying stabilities between the modified derivatives were also observed in different subcellular fractions of MCF-7 cells. Here the difference was not only observed among the modified oligonucleotides, but also between the various subcellular fractions as well. It is unclear what is the reason the greater susceptibility of the modified derivatives in the nuclear fraction compared to the other fraction. The increased stability of modified oligonucleotides in the post-nuclear fraction and the rapid hydrolysis of PO_2 oligonucleotide in the same fraction suggests the possibilty of more exonuclease activity in that fraction. Hoke *et al.* (40) have demonstrated the increased relative stability of a POS-derivative in both the nuclear and the post-nuclear fraction of HeLa cells. We found 22 and 50% of the initial concentration of the POS-derivative was hydrolyzed in 6 hrs by post-nuclear and nuclear fractions respectively. This discrepancy may result from the difference in enzyme content in the cell fractions of the two cell types. Caruthers *et al.* (41), however, have suggested absolute stability of PS_2-derivatives in HeLa cell extracts, but neither of these studies made a quantitative evaluation of such stability nor have they described the preparation or type of cell fraction in great detail. In contrast, we found that a significant amount (75−80%) of the PS_2-derivative was hydrolyzed in 6 hrs by either of these fractions. Differences in enzyme content or activity in the preparation could explain such a discrepancy in hydrolysis. Enzymes tend to lose their activity or become denatured unless proper care has been taken during the process of preparation of cell extracts, and this is particularly true when detergent is present in the media used for extraction. In our earlier work with peroxisomal enzyme, we have observed tremendous loss of enzyme activity in presence of the improper concentration of detergents (Ghosh and Hajra, unpublished results). It is unclear at this moment why the PS_2-derivative is less stable than the POS-derivative in these systems. Further studies with other enzymes and cell systems are warranted.

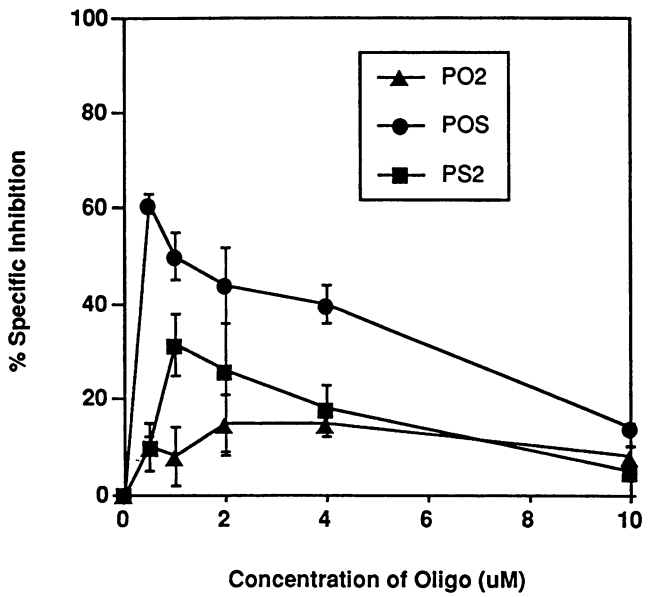

Figure 6. Specific translation inhibitions of rabbit β-globin mRNA in WGA at different concentrations of oligos.

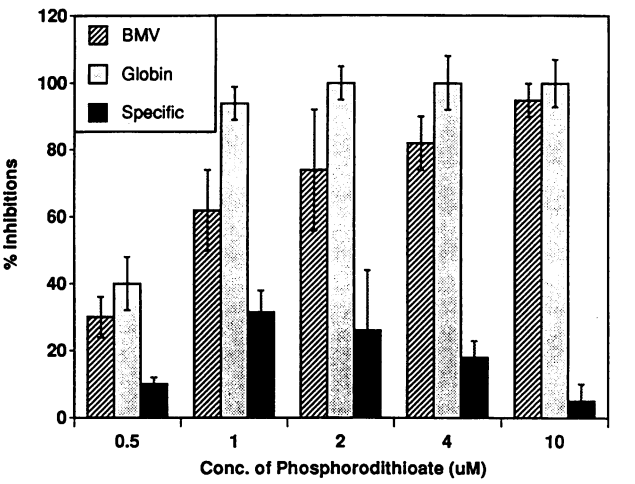

Figure 7. Translation inhibition of rabbit β-globin mRNA and BMV mRNA versus concentration of phosphorodithioate anti-β-globin 17-mer oligonucleotide. The specific inhibitions are the differences of the first two columns.

Although the cleavage of the target mRNA by RNase H in the PS_2 oligonucleotide−mRNA duplex was the same as the PO_2−mRNA duplex, the dithio-derivative showed slightly increased translation inhibition of the target mRNA compared to that of the PO_2 oligonucleotide in WGA. This was found only at lower concentrations of the oligonucleotide, and unlike the PO_2 oligonucleotide, the percentage of specific inhibition decreased with increased concentrations of the PS_2-derivative in the assay system. The translation inhibition could occur by interaction with the protein factors responsible for translation in the cell-free system. The lack of binding of the PS_2-derivative either with HSA or with protein factors present in the WGA extract ruled out the possibility of an interaction between the oligonucleotides and the protein factors present in the extract. In fact, none of the oligonucleotides showed any appreciable binding with the cell-free extract components, in a study conducted in a similar way as done with HSA. Two different membranes having cut-off values of 10 and 30K were used during these studies with the cell-free-system to ensure retention of any factors having molecular weight larger than 10,000 or 30,000. Even if there is any binding with some factor(s) present in the cell-free system (beyond our detection limit), the binding with a particular oligonucleotide will always be the same irrespective of different messenger molecules used for translation in the same cell-free system. Translation inhibition could, therefore, only be accounted for by cleavage of the target mRNA by RNase H.

Cleavage of the target mRNA in the oligo−mRNA duplex by RNase H requires prior hybridization, and this may result from both specific and non-specific hybridization. In either case, the hydrolysis of the mRNA would occur, and this will be reflected in the translation inhibition. A non-related mRNA like BMV mRNA was used to determine such non-specific hybridization, as a search in GenBank for BMV mRNA gave no homology with either the sense or antisense sequences with up to three mismatches (more than three mismatches gives negligible binding). In most cases the same target, but different oligos such as mismatch and random sequences, have been used as controls. We chose to carry out the translation studies this way because we felt that in terms of the actual therapeutic situation this is a more realistic control (37). A significant translation inhibition of the BMV mRNA particularly with the PS_2-derivative suggests an increased non-specific hybridization compared to the PO_2- or POS-derivatives. Even at 2 mM concentration, the PS_2-derivative produced 74% inhibition of the BMV mRNA compared to only 34% inhibition by the POS-derivative (Figure 6). Such non-specific hybridization is expected to occur also with the target mRNA, and thus the percent translation inhibition would be the sum of both specific and non-specific translation inhibition. The difference in specific translation inhibition between the PO_2- and PS_2-derivatives is not very significant, and they showed an equal *in-vitro* cleavage of the target mRNA by RNase H.

Although the replacement of both non-bridging oxygen atoms by sulphur atoms eliminates the problem of chirality, improves stability towards exonucleases, and reduces binding to proteins, the stability towards DNase I is quite poor. The present studies have been performed with pure nucleases, and the validity of this susceptibility may raise some questions since the cell does not contain such high concentrations of enzymes. However, the susceptibility of PS_2 in cellular extracts clearly demonstrates that this derivative is not as stable as the POS derivative. Besides, the modification also reduces hybridization with the target mRNA and produces higher non-specific translation inhibition. Thus, the results of the present study indicate that the PS_2 oligonucleotide offer very little advantage over the POS-derivative with respect to the properties required for antisense applications.

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